Scatter Factor/Hepatocyte Growth Factor Protects against Cytotoxic Death in Human Glioblastoma via Phosphatidylinositol 3-Kinase- and AKT-dependent Pathways

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INTRODUCTION

High-grade malignant gliomas are the most common cancers of the central nervous system. Despite treatment with surgical resection, radiation therapy, and chemotherapy, the mean overall survival is <1.5 years, and few patients survive for >3 years. A common reason for treatment failure is their innate resistance to radiation and chemotherapy. The mechanisms by which tumor cells survive death induced by these cytotoxic treatment modalities are becoming increasingly defined and involve growth/angiogenic factors, cytokines, and a diverse array of parallel and overlapping signaling pathways involved in the regulation of DNA repair and apoptosis. Identifying and ultimately targeting the mechanisms by which gliomas resist cytotoxic agents is likely to have a substantial impact on future treatment strategies.

ABSTRACT

We have shown recently that the multifunctional growth factor, scatter factor/hepatocyte growth factor (SF/HGF), and its receptor c-met enhance the malignancy of human glioblastoma through an autocrine stimulatory loop (R. Abounader et al., J. Natl. Cancer Inst., 91: 1548–1556, 1999). This report examines the effects of SF/HGF:c-met signaling on human glioma cell responses to DNA-damaging agents. Pretreating U373 human glioblastoma cells with recombinant SF/HGF partially abrogated their cytotoxic responses to gamma irradiation, cisplatin, camptothecin, Adriamycin, and Taxol in vitro. This cytoprotective effect of SF/HGF occurred at least in part through an inhibition of apoptosis, as evidenced by diminished terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling index and reduced DNA laddering. Anti-c-met U1/ribozyme gene transfer inhibited the ability of SF/HGF to protect against single-strand DNA breakage, DNA fragmentation, and glioblastoma cell death caused by DNA-damaging agents, demonstrating a requirement for c-met receptor function. Phosphorylation of the cell survival-promoting kinase Akt (protein kinase B) resulted from SF/HGF treatment of U373 cells, and both Akt phosphorylation and cell survival induced by SF/HGF were inhibited by phosphatidylinositol 3-kinase inhibitors but not by inhibitors of mitogen-activated protein kinase kinase or protein kinase C. Cytoprotection by SF/HGF in vitro also was inhibited by transient expression of dominant-negative Akt. Transgenic SF/HGF expression by intracranial 9L gliosarcomas reduced tumor cell sensitivity to gamma irradiation, confirming the cytoprotective effect of SF/HGF in vivo. These findings demonstrate that c-met receptor activation by SF/HGF protects certain glioblastoma cells from DNA-damaging agents by activating phosphatidylinositol 3-kinase-dependent and Akt-dependent antiapoptotic pathways.

SF,3 also known as HGF, is a multifunctional M, 90,000 heterodimeric growth factor, motility factor, and morphogen that, through its transmembrane tyrosine kinase receptor, the product of the c-met proto-oncogene, regulates developmental, regenerative, and neoplastic processes (1–5). SF/HGF and c-met are aberrantly expressed by a variety of cancers, including malignant gliomas, renal cell carcinomas, breast carcinomas, and sarcomas (6–10). In cancer cells, excessive c-met activation has been shown to induce transformation, tumor growth, tumor cell motility, invasion of extracellular matrices, and angiogenesis (6, 7, 11–14). SF/HGF:c-met signaling has also been found recently to function as a survival pathway and an inhibitor of apoptosis. SF/HGF protects carcinoma cells from apoptosis induced by DNA-damaging agents (15, 16), and expression of constitutively active c-met inhibits hepatocyte apoptosis (17). The antiapoptotic proteins BAG-1 (18) and Bcl-XL (16) have been implicated in the cytoprotective mechanisms of SF/HGF in carcinoma cell lines.

Human malignant gliomas frequently express both SF/HGF and c-met (11, 19, 20), which can establish an autocrine loop of biological significance (21). Glioma c-met expression correlates with glioma grade (20), and an analysis of human tumor specimens showed that malignant gliomas have a 7-fold higher SF/HGF content than low-grade gliomas (22). SF/HGF has been shown to promote dissemination of glioma cells, glioma cell proliferation, and glioma-associated angiogenesis (8, 14, 19). Inhibiting the expression of endogenous SF/HGF or c-met in human glioblastoma cells can reverse their malignant phenotype in vitro and in vivo (21). A role for SF/HGF:c-met signaling in the chemoresistance and radioresistance of malignant gliomas has not been established.

This study uses a human malignant glioma and a rat gliosarcoma cell line to show that SF/HGF can inhibit glioma cytotoxicity and apoptosis induced by either radiation or chemotherapeutic agents in vitro and in vivo. Inhibition of c-met gene expression by U1/ribozyme gene transfer demonstrated that the cytoprotective action of SF/HGF is c-met dependent. Furthermore, we show that SF/HGF activates the antiapoptotic factor Akt and that the cytoprotective action of SF/HGF is dependent, in part, upon Akt as well as on phosphatidylinositol 3'-kinase upstream from Akt activation. To our knowledge, this is the first description of an endogenous cytokine/growth factor that functions as a cytoprotective agent by acting directly on glioma cells.

MATERIALS AND METHODS

Cell Culture. Human U-373 MG (U-373) glioma cells were originally obtained from American Type Culture Collection (Rockville, MD). U-373 cell lines stably transfected with chimeric U1 small nuclear RNA/ribozyme constructs designed to inhibit endogenous c-met expression (designated U373-

3 The abbreviations used are: SF, scatter factor; HGF, hepatocyte growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PI3-kinase, phosphatidylinositol 3-kinase.
MET-KD) or with control plasmids (designated U373-CT) were established and characterized in detail as described previously (21). Stable control-transfected rat 9L gliosarcoma cells (9L-neo) and 9L cells transfected with human SF/HGF cDNA (9L-SF) were derived and characterized as described previously (14). All U-373 and 9L cell lines were cultured in DMEM (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), 2 mM L-glutamine, and gentamicin (50 mg/ml) in a humidified incubator at 37°C containing 5% CO2/95% air. Medium for stably transfected U-373 cells was supplemented with 100 μM/ml zeocyn (Invitrogen, Calabasa, CA), and medium for transfected 9L cells was supplemented with 300 μM/ml G418 (Life Technologies, Inc.).

**Expression Vectors and Transient Cell Transfections.** The Akt expression vectors (23) were kindly provided by Dr. Michael J. Quon (National Heart, Lung, and Blood Institute, NIH, Bethesda, MD). These include mammalian expression vectors encoding wild-type Akt, constitutively active Akt (Akt-yr3), a kinase-inactivated, dominant-negative Akt (Akt1K79A), and the empty expression vector pCIS2. Transient transfection was performed by incubating proliferating cells at ~50% of confluency overnight with 10 μg of plasmid DNA per 100 mm dish in the presence of Lipofectamine (Life Technologies, Inc.). Cells were then rinsed and incubated with complete medium for an additional 24 h prior to trypsinization and plating into 96-well plates for MTT cytotoxicity assays.

**MTT Cytotoxicity Assay.** U-373 cells were plated at 5 × 10² cells/well in groups of 8–10 in 96-well culture plates, in medium containing recombinant human SF/HGF (a kind gift of Genentech, Inc., S. Francisco, CA) at the indicated concentrations. PBS only was used as control. After 48 h, cells were exposed to the indicated concentrations of either Adriamycin (Sigma Corp., St. Louis, MO) for 2 h, paclitaxel (Sigma) for 2 h, cisplatin (Sigma) for 2 h, camptothecin (Sigma) for 48 h, or to ionizing radiation (radiation source = 132Cesium GammaCell Irradiator; Atomic Energy of Canada, Ltd., Mississauga, Ontario, Canada) in a single fraction. After 48 additional h from the time of starting the exposure to each cytotoxic agent, MTT (1 mg/ml; Sigma) was added to each well and incubated for 2 h at 37°C. The medium was then removed, and 100 μl of DMSO were added to each well. Absorbance at 570 nm was measured by a microculture plate reader (Dynatech; Alexandria, VA). Mean cell survival was calculated by absorbance units of samples of treated cells/mean absorbance units of samples of control cells.

**In Vivo Cytotoxicity.** 9L-SF or control 9L-neo (10⁵ cells/animal) were implanted by stereotactic injection into the cedrate-putamen of male Fischer 344 rats (weight, 200–250 g) as described previously (14). Seven days after injection of tumor cells, restrained and air-breathing animals were positioned with a fixed distance from a calibrated linear accelerator (Varian, Palo Alto, CA). Using 10 MeV energy, animals received 2000 cGy cranial irradiation via vertex port alignment in a single fraction as described previously (24). Animals were anesthetized and sacrificed 18 h later by intracardiac perfusion with sterile PBS, followed by 4% paraformaldehyde in PBS. Brains were removed, and serial 16-mm sections of tumors were cut, mounted on glass slides, and labeled by TUNEL immunohistochemistry as described below.

**TUNEL Immunocytochemistry.** TUNEL immunocytochemistry (25) was performed using the Apoptag In Situ Apoptosis Detection kit (Intergen Company, Purchase, NY). Briefly, U-373 cells were grown in 10-cm dishes in medium containing SF/HGF (10 ng/ml) or solvent only as control for 48 h. The cells were then exposed to either chemotherapeutic or ionizing radiation as described above. Eighteen h later, the cell medium was collected, and the remaining adherent cells were incubated with 0.25% trypsin. The suspended cells and medium were pooled and transferred to glass slides by cytosin centrifugation. Tumor tissue sections (16 μm thick) were obtained as described above. Cells and tissue sections were permeabilized with cold ethanol:acetic acid, 2:1 (v:v), and 0.1% Triton X-100 (Sigma) for 5 min. In tumor tissue sections, endogenous peroxidase was quenched with 3.0% hydrogen peroxide for 10 min. Immunocytochemistry was performed by sequentially adding terminal deoxynucleotidyl transferase, digoxigenin-dUTP, anti-digoxigenin antibody conjugated to peroxidase, and diaminoenzidine (Sigma). The specimens were then counterstained with methyl green and quantified as a digitized image using an automated KS 400 imaging system (Carl Zeiss, Eching bei Munchen, Germany). The number of TUNEL-positive cells among ~5000 cells/slide were determined. The fraction of total cells found to label by TUNEL immunocytochemistry was expressed as the apoptotic index.

**Preparation of Cell Protein Extracts.** Control cells or cells treated with SF/HGF in the presence or absence of Adriamycin (15 μM) and wortmannin (50 μM) were washed three times in PBS and placed on ice. Next, 900 ml of RIPA buffer (1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing fresh protease inhibitors (Calbiochem, San Diego, CA) were added to the dishes. The cell layers were then scraped to recover cell lysates. The lysates were then transferred to microcentrifuge tubes, incubated on ice for 1 h, and centrifuged at 15,000 × g at 4°C for 20 min. The supernatants were collected and concentrated using Centrulips-10 centrifugation filtration devices (Millipore, Bedford, MA). For analysis of c-met protein levels in U373-CT and in U373-Met-KD cell lines, total cell extracts were obtained from subconfluent cell monolayers using RIPA buffer containing protease inhibitors and centrifuged as described above. Western immunoblot analysis of samples was performed as described below.

**Western Blot Analysis.** SDS-PAGE and immunoblotting were performed according to the methods of Towbin et al. (26) with modifications. Cell extracts containing 20 μg of protein were diluted in Laemmli buffer containing β-mercaptoethanol and heated to 100°C for 2 min. The proteins were separated by SDS-PAGE at 50 mA for 20 min. Proteins were then electrophotographed transferred to Hybond ECL nitrocellulose membranes (Amersham Life Science, Buckinghamshire, England). The membranes were incubated with 5% nonfat dried milk in 1× Tris-buffered saline (100 mM Tris-HCl, 0.9% NaCl, pH 7.4) for 1 h and then incubated with a 1:1000 dilution of either rabbit polyclonal anti-Akt or anti-phosphorylated Akt (Ser473) IgG (New England Biolabs, Beverly, MA) overnight at 4°C. After washing, the membranes were incubated for 1 h with a 1:1000 dilution of horseradish peroxidase-conjugated goat antirabbit IgG (Jackson ImmunoResearch, West Grove, PA). The membrane-bound antibodies were then visualized using an ECL Western Blotting Detection kit (Amersham) on ECL Film. The films were digitized and quantitatively analyzed by densitometry (Molecular Dynamics, Sunnyvale, CA).

**DNA Fragmentation Assay.** Chromosomal DNA fragmentation in response to cytotoxic agents was determined by agarose gel electrophoresis as described previously (16, 27). Control cells or cells treated with Adriamycin (15 μM) in the presence or absence of SF/HGF were lysed in a solution containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 1% NP40. The supernatants were incubated with 1% SDS and 2.5 mg/ml RNase A for 2 h at 37°C and then incubated overnight with 2.5 mg/ml proteinase K at 56°C. DNA was precipitated with ammonium acetate and 95% ethanol. DNA was resuspended in electrophoresis loading buffer and electrophotographically separated in a 4% agarose gel. The DNA was stained with ethidium bromide and visualized using a transilluminator.
SF/HGF Protects Glioblastoma Cells from DNA-damaging Agents in Vitro. A microtiter spectrophotometric assay that measures the capacity of mitochondria to convert tetrazolium bromide (MTT) to formazan was used to quantify the relative survival of U-373 glioblastoma cells exposed to cytotoxic agents in the presence or absence of SF/HGF. Pretreatment of cells for 48 h with purified recombinant SF/HGF significantly increased the fraction of U-373 cells surviving exposure to either ionizing gamma radiation, the DNA cross-linking agent cisplatin (100 μM), or the topoisomerase I inhibitor, camptothecin (20 μM). The cytoprotective effects of SF/HGF were concentration dependent with statistically significant increases in cell survival occurring at SF/HGF concentrations ≥1.0 ng/ml. SF/HGF (3.0 ng/ml) increased the fraction of cells that survived exposure to either 5.0 Gy gamma irradiation, cisplatin, or camptothecin by 33% (P < 0.05), 77% (P < 0.01), and 85% (P < 0.001), respectively (Fig. 1). Preincubation of cells with SF/HGF was required for cytoprotection because treating cells with SF/HGF only during their exposure to DNA-damaging agents had no effect on cell survival (not shown).

SF/HGF Reduces Glioblastoma Cell Apoptosis Induced by DNA-damaging Agents in Vitro and in Vivo. The effect of SF/HGF on the fraction of cells undergoing apoptosis in response to cytotoxic agents in vitro was examined by TUNEL immunocytochemistry. In the absence of SF/HGF, 3.3% of U-373 cells became TUNEL positive after 5.0 Gy gamma irradiation; 6.8% of cells became TUNEL positive after exposure to cisplatin (30 μM), and 2.9% became TUNEL positive after exposure to camptothecin (20 μM). Fewer than 0.1% of cells were TUNEL positive in control cultures exposed to buffer only under identical conditions. SF/HGF reduced the fraction of apoptotic nuclei after exposure to either chemotherapy or ionizing gamma radiation. Pretreatment of cells with 3.0 ng/ml recombinant human SF/HGF resulted in a 38% reduction in the fraction of TUNEL-positive cells exposed to radiation (P < 0.001), a 35% reduction of TUNEL-positive cells exposed to cisplatin (P < 0.001), and a 79% reduction of TUNEL-positive cells exposed to camptothecin (P < 0.001; Fig. 2, A and B).

Human SF/HGF gene transfer was used to examine the effect of SF/HGF production by glioma cells on radiation-induced apoptosis in vivo. We chose the 9L gliosarcoma model for these experiments because 9L cells generate more rapidly growing and reproducible tumors than U-373 cells. However, like U-373 cells, 9L cells normally express the SF/HGF receptor c-met but lack detectable SF/HGF expression (13). Stable control-transfected SF/HGF-negative 9L cells (9L-neo) and 9L cells engineered to secrete human SF/HGF (9L-SF) were established and characterized as reported previously (13). 9L-neo and 9L-SF cells were stereotactically implanted to caudate-putamen of syngeneic Fischer 344 rats to produce control SF/HGF-inhibited glioma cell apoptosis in vitro and in vivo. A, U-373 cells were pretreated with SF/HGF for 48 h and then exposed to either ionizing radiation, cisplatin (CDDP, 30 μM), or camptothecin (CPT, 20 μM). Eighteen h later, cells floating in the medium and cells trypsinized from the monolayer were pooled, transferred to glass slides by cytospin centrifugation, labeled by TUNEL immunocytochemistry, and counterstained with methyl green. Apoptotic indices were calculated as the ratio of TUNEL-labeled cells to total cells. Cells pretreated with SF/HGF had significantly lower apoptotic indices relative to controls for each cytotoxic agent examined (means; bars, SE; P < 0.001). B, representative light photomicrographs of U-373 tumor cells demonstrating TUNEL labeling (dark labeling most abundant in lower left panel). C, Fischer rats bearing intracranial tumors established by stereotactically injecting SF/HGF-negative control-transfected 9L gliosarcoma cells (9L-neo) or 9L cells engineered to secrete human SF/HGF (9L-SF) received 2.0 Gy cranial irradiation or no irradiation as control (n = 4). Eighteen h later, animals were sacrificed, brains were removed, and histological tumor sections were labeled by TUNEL immunohistochemistry. Apoptotic indices were calculated as the ratio of TUNEL-labeled cells/total cells. The apoptotic index of irradiated 9L-SF tumors was significantly less than that of irradiated control tumors (means; bars, SE; P < 0.001). Nonirradiated 9L-neo and 9L-SF tumors did not differ in their apoptotic indices that were <0.025% (not shown).
negative and SF/HGF-positive brain tumors, respectively (14). Seven days later, animals bearing established tumors were exposed to 2.0 Gy cranial irradiation in a single vertex fraction, or they were sham-irradiated as control. Animals were perfusion fixed 24 h later, and tumor sections were analyzed for tumor cell apoptosis by TUNEL immunohistochemistry. The percentage of TUNEL-positive cells found in unirradiated tumors was <0.025% and did not differ between control 9L-neo and 9L-SF tumors. Radiation increased the percentage of TUNEL-positive cells in the 9L-neo tumors by >25-fold to 0.67%. In contrast, the percentage of TUNEL-positive cells in the irradiated 9L-SF tumors increased to only 0.23%, approximately one-third of that seen in the irradiated control tumors (P < 0.001; Fig. 2C). Anti-c-met Ribozyme Inhibits Cytoprotection by SF/HGF. To determine the role of the c-met receptor in the cytoprotective action of SF/HGF, we examined the cytoprotective effects of SF/HGF in control-transfected U-373 cells (U373-CT) and in U-373 cells stably transfected with a chimeric U1/ribozyme previously shown to specifically inhibit (i.e., knock-down) human c-met expression (designated U373-Met-KD; Ref. 21). Extracts from U373-Met-KD cell lines were found to contain approximately 5–11% of c-met mRNA and approximately 2–23% of c-met protein when compared with U373-CT cells, as determined by Northern hybridization and immunoblotting, respectively (Fig. 3; Ref. 21).

The effect of SF/HGF on the response of U373-CT cells and U373-Met-KD cells to DNA-damaging agents was examined using three distinct end points of cell injury and DNA damage. As expected based on the results described above, preincubation of U373-CT cells with SF/HGF increased the fraction of cells surviving incubation with Adriamycin, cisplatin, and Taxol as determined by MTT assay by 114, 68, and 76%, respectively. In contrast, SF/HGF enhanced the survival of U373-Met-KD cell lines after their exposure to the same cytotoxic agents by only 21, 18, and 42%, respectively (Fig. 4A). Thus, anti-c-met ribozyme reduced SF/HGF-mediated cytoprotection by approximately 45–80% (P < 0.001). Likewise, SF/HGF substantially inhibited Adriamycin-induced DNA laddering in U373-CT cells but had little effect on this marker of apoptosis in U373-Met-KD cells (Fig. 4B).

SF/HGF PROTECTS GLIOMAS FROM CELL DEATH

**Fig. 3. Inhibition of c-met expression in U-373 cells by U1/ribozymes.** Wild-type U-373 cells were stably transfected with control plasmid or with plasmid bearing a transgene coding for anti-c-met U1/ribozyme as described in “Materials and Methods.” A, total cellular RNA derived from control-transfected cell lines (CT) and from three U1/ribozyme-transfected cell lines (KD) was analyzed by Northern hybridization using c-met and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. B, total cellular protein obtained from the same control-transfected cell lines and U1/ribozyme-transfected cell lines was analyzed by immunoblot analysis (10 μg total protein/lane) using anti-c-met antibodies. Specific antibody binding to membrane was detected by chemiluminescence. Substantial knock-down (KD) of c-met expression resulted from U1/ribozyme transfection as evidenced by reductions in c-met RNA relative to GAPDH and by reductions in immunoreactive c-met protein.
Ribozyme inhibition of c-met expression completely reversed the ability of SF/HGF to protect glioblastoma cells from chemotherapy-induced DNA single-strand breakage as determined by DNA filter elution assay (Fig. 4C).

SF/HGF Cytoprotection Is PI3-Kinase Dependent and Akt Dependent. PI3-Kinase-dependent activation of Akt has emerged recently as an important mechanism by which malignant cells gain resistance to cytotoxic agents (29, 30). We asked whether SF/HGF increases Akt activation in glioblastoma cells using polyclonal antibodies that specifically recognize either the activated form of Akt (phosphorylated at serine residue 473) or total Akt. Incubating U-373 cells with SF/HGF for 2-48 h resulted in an ~5-fold increase in the phosphorylated form of c-Akt protein relative to total cellular Akt (Fig. 5). Incubation of U-373 cells with either wortmannin (50 nM) or LY249002 (10 μM), distinct selective inhibitors of PI3-kinase, completely inhibited the activation of Akt by SF/HGF (Fig. 5). In contrast, Akt activation by SF/HGF was minimally effected by treatment with PD98059 (30 μM), a mitogen-activated protein kinase kinase inhibitor, or with GF109302X (1 μM), a protein kinase C inhibitor (Fig. 5).

We next asked whether inhibition of PI3-kinase, under conditions that block SF/HGF-mediated Akt activation, altered the cytoprotective action of SF/HGF in U-373 cells. Cells were pretreated with SF/HGF in the presence or absence of either wortmannin or LY294002 and then exposed to camptothecin as described earlier. At the concentrations used in these experiments, neither wortmannin nor LY294002 altered the basal viability of U373 cells. LY294002 and wortmannin inhibited the capacity of SF/HGF to increase cell survival by approximately 80 and 50%, respectively (Fig. 6A). Neither PD98059, the mitogen-activated protein kinase kinase inhibitor, nor GF109302X, the protein kinase C inhibitor, altered SF/HGF-mediated cytoprotection. To directly examine the role of c-Akt in SF/HGF cytoprotection, U373 cells were transiently transfected with either a dominant-negative kinase-inactivated Akt (DNAkt), constitutively activated Akt (Akt-myr), wild-type Akt, or empty expression vector as control (all kindly provided by Dr. Michael Quon, NIH, Bethesda, MD) prior to treatment with SF/HGF and/or camptothecin (Fig. 6B). Expression of wild-type Akt reduced the cytotoxicity of camptothecin by ~50%, but these cells remained sensitive to the cytoprotective action of SF/HGF. Expression of constitutively active Akt completely blocked the cytotoxicity of camptothecin. Expression of dominant-negative Akt had no significant effect on the cytotoxicity of camptothecin but inhibited the cytoprotective action of SF/HGF by ~50%.

**DISCUSSION**

Tumors gain resistance to cytotoxic agents as a result of several different molecular mechanisms. These include enhanced DNA repair, multidrug resistance associated protein-mediated exportation of cytotoxic drugs, alterations in intracellular glutathione and metallo-thionine concentrations, increased expression of aldehyde dehydrogenase and O6-alkylguanine-DNA-alkyltransferase, and structural alteration of microtubules (31, 32). Furthermore, mutations in the p53 tumor suppressor gene and alterations in the levels of topoisomerase may also contribute to the induction of chemotherapy and radiation therapy-resistant phenotypes (33). Finally, expression of members of the Bcl family of apoptosis regulating proteins and the interleukin-1β-converting enzyme-like family of cysteine proteases (also termed caspases) influence the susceptibility of tumors to apoptosis (34). Growth factors, such as acidic and basic fibroblast growth factor, insulin-like growth factor-I, and cytokines are able to protect certain tumor cell lines from cytotoxic cell death (35, 36). Better understanding of these mechanisms will lead to new strategies to inhibit tumor cell resistance to existing cytotoxic therapies.

We show in this report that the multifunctional growth factor and angiogenic factor SF/HGF, which has recently been implicated in glioma malignancy, decreases cell death, apoptosis, and DNA strand
breakage induced by cytotoxic agents in the human U-373 malignant glioma cell line in vitro. Importantly, we also demonstrate that transgenic SF/HGF expression in intracranial rat 9L gliomas inhibits their apoptotic response to gamma irradiation in vivo. These findings are consistent with the cytoprotective action of SF/HGF reported recently in other systemic tumor cell lines and in nonneoplastic cells. Recently, we showed that SF/HGF blocks the induction of cell death and apoptosis by various DNA-damaging agents, including chemotherapy, ionizing radiation, and UV in human breast cancer cells and mouse mammary tumor cells (16). Renal epithelial cells transfected to produce SF/HGF are more resistant to cisplatin-induced apoptosis (37). Furthermore, SF/HGF protects several epithelial cell types from apoptosis-induced by detachment from their adhesive substrate (i.e., anoikis) or by the protein kinase inhibitor, staurosporine (18, 38).

Finally, bone marrow stem cells treated with SF/HGF are more resistant to ionizing radiation (39). Our findings in this report, in conjunction with the association of elevated levels of SF/HGF and its receptor c-met in malignant versus low-grade human glioma specimens (9, 20, 22, 40), strongly implicate a role for endogenous SF/HGF in the resistance of high-grade gliomas to radiation- and chemotherapy-induced cell death. To our knowledge, SF/HGF is the first growth factor/cytokine reported to inhibit cytotoxic agent-induced apoptosis by direct autocrine action on glioma cells.

The cytotoxic agents used in these experiments have different mechanisms of action. Ionizing radiation induces formation of free oxygen radicals leading to single and double DNA strand breaks (41). Cisplatin forms covalent adducts between adjacent guanine residues in DNA, depressing DNA synthesis and causing a transient block between G1-G2 and S-phase (42, 43). Camptothecin inhibits topoisomerase I through formation of stable topoisomerase I-DNA cleavable complexes (7). Taxol (paclitaxel) binds to the beta-subunit of tubulin, thereby stabilizing tubulin and blocking cell cycle progression through mitosis (44). Adriamycin has multiple mechanisms of action that include direct intercalation and covalent binding to DNA and inhibition of topoisomerases. That SF/HGF is cytoprotective against such an array of agents suggests a mechanism of action involving pathways such as DNA repair and antiapoptosis that are fundamental to cell evasion of death signals. We demonstrate in U-373 human malignant glioma cells that SF/HGF activates the PI3-kinase/Akt pathway that mediates growth factor-induced cell survival and suppresses apoptotic death induced by various stimuli, including growth factor withdrawal, cell cycle discordance, loss of cell adhesion, and DNA damage in many cell types. In vitro cytoprotection was completely inhibited by two distinct PI3-kinase inhibitors. PI3-kinase activates a number of cell signaling pathways with cytoprotective functions. Akt is activated by PI3-kinase through phospholipid binding and phosphorylation at Thr308 and Ser473 (45). We show that SF/HGF stimulates a substantial increase in active phospho-Akt, and we implicate Akt in ~50% of SF/HGF-mediated cytoprotection. We have recently reported a similar mechanism for SF/HGF-mediated cytoprotection in carcinoma cells (49). The mechanism downstream from Akt by which SF/HGF induces resistance to cytotoxic agents has not yet been defined but may result, in part, from influences on members of the Bcl family of apoptosis-regulating proteins, including Bcl-2, Bcl-XL, or BAG-1. Activated Akt phosphorylates Bad, a distant member of the Bcl-2 apoptosis-regulating family of proteins. Bad phosphorylation activates cell survival promoting pathways mediated by proteins such as Bcl-2 and Bcl-XL (30, 46). Recently, we reported that SF/HGF inhibits the ability of Adriamycin to down-regulate the antiapoptosis protein, Bcl-XL, in MDA-MB-453 carcinoma cells (16). Furthermore, Bardelli et al. (18) reported binding of the antiapoptotic protein, BAG-1, with the SF/HGF receptor c-met, and overexpression of BAG-1 enhances hepatocyte progenitor cell survival from apoptosis. In addition, activation of Akt by SF/HGF could result in the phosphorylation of pro-caspase-9 at Ser196 and thereby promote cell survival by inhibiting its proteolytic activity (47). Our finding that dominant-negative Akt only partially reverses SF/HGF cytoprotection suggests a potential role for a PI3-kinase-dependent but Akt-independent pathway that is presently being explored.

In conclusion, we establish in this report that SF/HGF functions as a glioma cell survival factor both in vitro and in vivo. A mechanism involving PI3-kinase and Akt is proposed based upon the in vitro analysis of the U-373 human malignant glioma cell line. Strategies developed recently to inhibit SF/HGF:c-met signaling by targeting
gene expression (21) or c-met receptor function (48) may be useful in enhancing the therapeutic response of malignant gliomas to existing cytotoxic modalities.

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REFERENCES


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